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Resistance of Purified Collagen to Degradation by Salt-Tolerant Bacteria in Pure Culture*

By A. L. EVERETT and T. C. CORDON

*Eastern Regional Research Laboratory***
Philadelphia 18, *Pennsylvania*

Skins from many kinds of animals may be converted into durable leather products of a thousand uses. Freshly removed skins are not considered a suitable raw material for the tanner because they are so highly perishable. Unavoidable delays between time of slaughter and the beginning of processing make it necessary to preserve hides and skins immediately after removal from the animal. Spoilage may be prevented by drying the skins, but in most countries it is customary to use a curing process employing strong solutions of sodium chloride. Salt, however, is not an ideal preservative. Under adverse conditions, and especially during hot weather, bacterial damage may occur and cause a loss of collagen, the actual leather-making substance of skin. This paper presents a study of specific collagen degradation at high levels of salt, employing pure culture conditions with bacteria isolated from hide-curing environments.

Curing may be done slowly, between layers of dry salt in a pack, or more rapidly by soaking in saturated brine. The primary function of the salt is to inhibit bacterial growth by a combination of effects such as dehydration, toxic action, enzyme inactivation, etc. Temperature and relative humidity are important factors. A secondary action is the partial removal of globular proteins from skin which not only encourage bacterial growth but also are undesirable for leather-making purposes.

After salt curing various forms of bacterial damage have commonly been observed. Most noticeable of these are the red or violet flesh stains, especially the condition described as "red heat". The organism responsible for such action are halophilic and are known to originate mainly from marine salt [11, 18]. Other types of bacteria normally present on the animal's skin at the time of slaughter may be of soil or intestinal origin. Those that possess salt tolerance

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and proteolytic activity may cause destruction of the skin substance or grain surface. Such organisms may be quite adaptable [1], enabling them to grow under changing concentrations of salt and moisture. Some interesting concepts to explain this behavior have recently been explored [16]. Chemical additives have been recommended [10, 21] to improve the preservative action of salt; or the numbers of bacteria may be greatly reduced by thoroughly washing the hides before curing [6].

Chemical methods for evaluating the extent of bacterial damage to hide proteins have usually been limited to measuring the various forms of soluble nitrogen liberated. Volatile nitrogen in particular has been advocated as the best criterion of curing conditions [9, 20]. However, since none of these methods was specific for collagen itself, they gave only indirect evidence of its degradation. Mammalian collagen, or the gelatin derived from it, is the only common protein containing a large amount of the amino acid hydroxyproline (13–14 per cent). Determination of this compound by the colorimetric procedure of Neuman and Logan [14] offers a specific method for estimating collagen in soluble or insoluble form.

A prime necessity for pure culture studies is a suitable sterilization method. This was a stumbling block for many years because of the extreme heat sensitivity of collagen. Finally, Maxwell [12] developed a chemical method for use with sheepskin based on the action of sulfurous acid. After modification as suggested by Anderson [2] to prevent swelling of the fibers, this has proven to be a very satisfactory treatment. There was no apparent effect on the properties of collagen.

Materials and Methods

Culture media. For isolating and maintaining cultures, nutrient agar (Difco) was enriched with yeast extract 0.6 per cent, tryptone 0.2 per cent, glucose 0.2 per cent and salt 12 to 23 per cent as indicated. For the screening and collagen tests a broth was prepared by dissolving the yeast, tryptone, glucose and salt in water*. A study of growth factor requirements by Katznelson and Lochhead [8] emphasized the value of yeast extract for stimulating growth.

Isolation and screening of cultures. Samples of used salt, brine and salted hide were obtained from commercial slaughterhouses; laboratory curing experiments and pieces of fresh hide also served as source materials. Salt-agar dilution plates prepared from these samples yielded a large collection of salt tolerant and halophilic bacteria. Duplicate plates without salt were used to isolate another group for adaptation experiments. Although most of the organisms were facultative, no obligate anaerobes could be found. After

*Ingredients such as peptone and gelatin were undesirable in the test medium because of their hydroxyproline content.

purification all cultures were screened for ability to liquefy 12 per cent gelatin. It is generally accepted that "collagenase" activity implies a similar action against gelatin [4]. With this assumption it was possible to eliminate the totally inactive isolates from further testing. Some of the cultures were screened also with commercial hide powder, but the results obtained were identical to those with gelatin. This confirms the experience of Grassmann [7] and many others concerning the denatured character of the collagen in hide powder.

As a result of screening a group of 67 salt tolerant cultures was selected for the collagen tests. No attempt was made to classify them beyond examination of stained preparations. Anderson [3] recently reviewed the literature concerning bacteria involved in red discolorations. Some of the cultures in our collection (groups D and E below) were of this type. They are notable for being true halophiles and sensitive to water or dilute salt solution. Other workers in the field have placed their isolates in recognized classes or genera [1 9]. But for our purpose, identification of the cultures was of no immediate concern.

Adaptation. Another group of 106 cultures isolated on salt-free media was adapted to growth with salt by repeated transfer to tubes of broth containing gradually increasing amounts of salt. A total of 75 strains attained good growth in 2 M salt (ca 12 per cent) and a number of these could also tolerate 2.5 M. However, the 3M level proved to be a critical point beyond which none of the adapted strains would grow. This behavior seems interesting in view of the fact that most of the cultures originated from saturated salt environments (above 5N). Since the majority were spore-forming rods it is probable that these types survived in the form of spores.

Collagens. Cowhide collagen was used as substrate for testing activity of the organisms. Only the center layer of hide was utilized since this is probably the purest natural form of collagen. One preparation had been treated according to commercial practice for limed hide; i. e., it was salt-cured, soaked in water and then in saturated lime liquor long enough to remove the hair. After drying and cutting into small pieces this was designated as "limed collagen". The other preparation was "unlimed collagen", treated mildly to avoid denaturation so that it resembled what is often referred to as "native" or purified collagen. It was extracted with salt solution, dilute lime water and solvent to remove practically all impurities.* The two preparations were directly compared in the tests, and differences may be attributed mainly to the action of lime.

Sterilization. Sterilization of collagen was accomplished essentially by the method of Maxwell [1 2]. Collagen pieces (5–10 g. lots) were treated in suc-

*This was a portion of sample 19 for amide nitrogen analysis by Mellon, Viola, and Hoover[13] of this laboratory, to whom we are indebted.

cession, in closed containers, with 100 ml. of each of the following solutions: (1) 0.025 M. sodium metabisulfite for 17 hr.; (2) 0.3 M hydrogen peroxide for 7 hr.; (3) sterile 0.1 M sodium bicarbonate for 17 hr.; (4) two changes of sterile 10 per cent salt. The bisulfite soln. (4.75 g/L) was adjusted to pH 2 with strong HCl before making to volume. The first three solutions also contained 10 per cent added salt to prevent swelling. After the stated times, each reagent was decanted and replaced by the next in the series. Finally, the treated pieces were transferred aseptically to tubes of sterile salt broth to form the complete medium. Anderson[2] recommends treating in buffer soln. after sterilization, but this was not found to be necessary, since final pH was about 6.8–7.0.

Test Procedure. Tubes of the complete medium, each containing 1 or 2 pieces of sterile collagen, were incubated for several weeks at 30°C. to check sterility and then inoculated with the test cultures. Incubation was continued in a moist chamber for 2 to 3 months before subjecting the materials to chemical analysis. Stuart[20] and others have emphasized the long lag period and slow growth rate characteristic of halophilic bacteria, which require extended incubation for proper evaluation of their activities.

In this study, degradation of collagen was measured by determining the relative amount converted to soluble form, as estimated by corresponding amounts of hydroxyproline. Soluble matter was separated by centrifugation, and the residues were washed once with warm water. Aliquots of the combined supernatants were used to prepare acid hydrolysates of the soluble fractions, by autoclaving with 6 *N* HCl in sealed tubes (20 lb. – 4 hr.). Insoluble residues were decomposed with warm acid, transferred to small tubes and hydrolyzed in the same manner.

Hydroxyproline was determined colorimetrically in the neutralized hydrolysates by the Neuman and Logan method[14].

$$\text{Per cent activity} = \frac{\text{mg. soluble hydroxyproline}}{\text{mg. total hydroxyproline}} \times 100,$$ where total hydroxyproline is merely the sum of the soluble and insoluble fractions. The range of values for uninoculated controls is included in the tables to show the extent of non-bacterial effects.

Results

Halophilic organisms. Single cultures of the 67 strains were first tested on both limed and unlimed collagen. Some representative results are shown in Table I. The organisms are grouped according to gram stain and morphology, with notations in the next columns on the requirement for salt. It might be added that many of them will tolerate complete saturation,

TABLE I

Salt Tolerance and Collagen Solubilizing Activity of Representative Single Cultures

Cultures	Growth without Salt	Growth with 4M Salt (23%)	Activity* in 15% Salt Broth + Substrate		
			12% Gelatin	Limed** Collagen	Unlimed*** Collagen
Uninoculated controls	—	—	0	10–20%	3–9%
Gram-pos. rods:					
No. 12	Yes	No	+++	62	8
No. 11	Yes	Yes	+++	32	5
No. 87	No	Yes	++	34	6
Gram-neg. rods:					
No. 21	Yes	Yes	+++	41	5
No. 22	Yes	Yes	+++	36	3
No. 20	No	Yes	++	20	4
Gram-pos. cocci:					
No. 28	No	Yes	++	98	5
No. 78	No	Yes	++	31	7
No. 59	Yes	Yes	+	25	7
Gram-var. red cocci:					
No. 112	No	Yes	+	20	6
Gram. var. irreg. rods:					
No. 104	No	Yes	++	20	10

*Gelatin, complete liquefaction: +++in 4 weeks; ++ 4 to 8 weeks; + over 8 weeks. Collagen, solubilization: hydroxyproline in solution as per cent of total found.

**Center layer of cowhide soaked in saturated lime liquor; chemically sterilized.

***Center layer of cowhide carefully purified without liming; chemically sterilized.

notably those requiring salt for growth. Uninoculated controls showed an appreciable amount of degradation of limed collagen, but none with the unlimed. Two of the cultures, No. 12 and No. 28, were highly active against limed collagen, and a number of others were moderately active. But none of the cultures showed any significant activity with unlimed collagen.

Table II gives some of the results of experiments with mixed cultures. The members of each morphological group were pooled and tested as group mixtures. Again there was moderate activity against limed collagen, but no definite evidence of enhanced or synergistic effect. And again there was no action on the unlimed collagen. Then the group mixtures were tested in all possible combinations with each other. A complete range of values was obtained, but lack of space prevents showing them all. The grand mixture of all 5 groups was consistently highest, averaging above 90 per cent activity on limed collagen but with insignificant action on the unlimed. In studying the results it was concluded that combinations containing group B, group E or both were the most active. Consequently further mixtures were prepared to narrow the effect. Two examples shown at the bottom of the table illustrate the enhanced activity of these mixtures against both substrates. This was

TABLE II

Enhanced Activity Shown by Various Mixed Cultures

Composition of Culture Mixtures	Total No. of strains	Activity* in 15% Salt Broth + Substrate	
		Limed** Collagen	Unlimed*** Collagen
Uninoculated controls	0	10–20%	3–9%
A Gram-pos. rods	19	47	10
B Gram-neg. rods	5	32	5
C Gram-pos. cocci	30	40	5
D Gram-var. red cocci	10	15	8
E Gram-var. irreg. rods	3	42	8
Group Combinations			
A + B	24	65	8
A + C	49	29	—
A + D	29	22	—
A + E	22	41	11
B + C	35	37	5
B + D	15	21	6
B + E	8	91	11
C + D	40	24	—
C + E	33	62	14
D + E	13	58	8
A + B + C + D + E	67	93	15
Selected Mixtures			
No. 19 (B) + E	4	88	36
No. 104 (E) + B	6	93	40

*Collagen solubilization: hydroxyproline in solution as per cent of total found.
**Center layer of cowhide soaked in saturated lime liquor; chemically sterilized.
***Center layer of cowhide carefully purified without liming; chemically sterilized.

the only occasion where we ever detected significant degradation of unlimed collagen in a salt medium.

Adapted organisms. As described above, a group of 75 cultures from salt-free media had been adapted to grow in the presence of 12 per cent salt. Table III lists some representative results when these organisms were tested before and after adaptation. Without salt most of these cultures were highly active against limed collagen. Also a number of them showed some action on the unlimed collagen in the absence of salt. However, when tested with 12 per cent salt after adaptation, activity sharply declined in almost every case. The important point is that all activity against unlimed collagen had disappeared.

Discussion

The results obtained indicate that purified collagen, largely unaltered by chemical or physical change, is quite resistant to bacterial attack in high

TABLE III

Influence of Salt Content on Activity of Salt-adapted Cultures

Cultures	Activity* in Broth with and without Salt + Substrate				
	Hide Powder No Salt	Limed** Collagen No Salt	Limed** Collagen 12% Salt	Unlimed*** Collagen No Salt	Unlimed*** Collagen 12% Salt
Uninoculated controls	0	4–14%	12–22%	1–2%	1–2%
Gram-pos. sporing rods					
No. 23	+++	98	87	12	5
No. 42	+++	100	46	20	4
No. 67	++	99	50	19	3
No. 69	++	99	22	32	3
No. 77	+++	100	17	57	3
Gram-pos. nonsporing rods					
No. 2	++	98	32	5	3
No. 3	++	98	29	12	3
No. 5	++	77	24	9	4
No. 10	++	97	29	10	4
Gram-pos. cocci					
No. 102	+	13	50	2	2
No. 106	+	27	13	8	2
Gram-neg. rods					
No. 105	++	37	59	2	2

*Hide powder, complete solubilization: +++ in 4 weeks; ++ 4 to 8 weeks; + over 8 weeks. Collagens, solubilization: Hydroxyproline in solution as per cent of total found.

**Center layer of cowhide soaked in saturated lime liquor; chemically sterilized.

***Center layer of cowhide carefully purified without liming; chemically sterilized.

salt media. Some members of the genus *Clostridium* have been shown to possess collagenase activity [15], as well as the enzymes papain [5] and pepsin [17]. But such agents would not be expected to operate in the presence of high salt concentration. Since curing damage does occur, however, other organisms may be a source of "collagenase", especially when present in suitable combinations and under favorable conditions. A more practical finding is confirmation of the importance of avoiding contact with denaturing effects such as moist heat and alkali.

Summary

Salt-tolerant and halophilic bacteria were isolated from salted hides. Those cultures able to liquefy gelatin were tested for solubilizing activity on limed and unlimed collagen. A modified chemical method was used to sterilize the collagen pieces without apparent alteration of properties. Bacterial degradation was measured by colorimetric estimation of the proportion of hydroxyproline in soluble form after suitable incubation.

Results were obtained for single cultures as well as various combinations of pooled mixtures. In general the unlimed or "native" collagen proved ex-

tremely resistant to attack, while many of the strains were moderately active on the limed material. In only a few cases, selected culture mixtures appeared to degrade both forms of collagen. Other tests with organisms adapted to salt tolerance showed the flexible nature of this adaptive property, and the effect of salt on their collagen-solubilizing activity. It was concluded that synergistic activity may well account for bacterial damage under curing conditions, and that alkaline denaturation greatly increases the susceptibility of collagen to bacterial attack.

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